昆 虫 学 报 *Acta Entomologica Sinica*, April 2004, 47(2): 229 – 235 ISSN 0454-6296

Characterizing populations of *Anoplophora glabripennis* and related taxa with RAPD *

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Abstract: Five *Anoplophora* sibling species and eight geographical populations of the Asian longhorned beetle (ALB), *Anoplophora glabripennis* (Motsch.) collected from China and the USA were analyzed using the random amplified polymorphic DNA (RAPD) method. A total of 51 random primers (20 OPH, 20 OPL, 11 OPQ kits) were selected, of the 40 primers used in primer group Ⅰ, 26 primers produced polymorphic bands in phylogeny analyses of *Anoplophora* species and *A. glabripennis* populations. In primer group Ⅱ, 19 of the 31 primers produced polymorphic bands in *A. glabripennis* population analyses. Based on the computer-generated RAPD cladogram using primer group Ⅰ, eight geographical populations of *A. glabripennis* and two populations of *A. nobilis* can be grouped in one phylogenic cluster that is different from the other *Anoplophora* species. Six geographical populations of *A. glabripennis* in China form a cluster branch, which can be divided into two sub-branches: one sub-branch consists of populations from the provinces of Shaanxi, Shandong, Hebei, Nei Mongol, and Ningxia, and the other consists of *A. glabripennis* population from Gansu Province. The two *A. glabripennis* populations from New York and Chicago can be considered as an independent branch of a cluster with 0.2525 genetic distances between them. Similar results were also obtained with *A. glabripennis* geographical populations using primer group Ⅱ. These results indicated that the specimens of *A. glabripennis* populations collected from the USA were somewhat different genetically from specimens collected from some parts of China. The cladogram showed that *A. nobilis* populations from Gansu and Ningxia were mixed with the *A. glabripennis* cluster with little difference between the two species, which strongly supports the findings that the two may in fact be a single species.

Key words: *Anoplophora*; *A. glabripennis*; *A. nobilis*; population; RAPD; genetic distance

1 INTRODUCTION

The Asian longhorned beetle (ALB), *Anoplophora glabripennis* (Motsch.) (Coleoptera: Cerambycidae) is native to parts of Asia. It is a major pest in north China, causing serious damage to poplars in shelt-belts and other tree species (Gao and Li, 2001). The beetle was initially discovered in several locations in North America in 1996, and has infested trees such as maple and elm, among others (Haack and Cavey, 1997; USDA APHIS, 1999; USDA APHIS PPQ, 2002; USDA FS, 2003). To prevent additional introductions of *A. glabripennis* and other wood-inhabiting pests, USDA-APHIS drafted an interim rule that requires treatments of imported logs, lumber, and other unmanufactured wood packing materials from China based on a certified treatment schedule (US Federal Register, 1998). North American *A. glabripennis* populations were likely founded beetles that hitch-hiked in wood packing materials from within their native range, which includes China, Japan and Korea. China is thought to be the most likely source of north American *A. glabripennis* infestation based on circumstantial evidences, including the high *A. glabripennis* population levels in China (compared with very low population levels in Korea and Japan), and numerous interceptions of *Anoplophora* spp. at the US ports in wood packing materials originated from China. It is not known, however, where the Chicago and New York population originates.

Randomly amplified polymorphic DNA (RAPD) is a DNA polymorphic detecting technique at large MW DNA level developed by Williams *et al*. (1990) and Welsh and MeClelland (1990). This technique has been widely used in genetic variation and phylogeny

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基金项目：国家质检总局立项、中美合作课题(国质检 K048-2000)

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收稿日期 Received: 2003-06-10; 接受日期 Accepted: 2003-11-28

analyses with great successes. Williams *et al*. (1994) used RAPD method to detect the DNA polymorphism among *Listronotus bonariensis* populations and indicated that the exotic weevil in New Zealand originated from the eastern coast of South American. Polymorphic bands generated using the RAPD were also found useful as genetic markers to study the inheritance, such as Schnell *et al*. (1996), An *et al*. (1998), Yang *et al*. (1999), Biron *et al*. (2000), Sun *et al*. (2000), Zhu *et al*. (2001), Jiang *et al*. (2002), etc. However, a literature search through AGRICOLA (National Agricultural Library, 2002) using key words "*Anoplophora*" and "RAPD" did not result in any published references at the time when this project was initiated. In the present paper, we report our results of genetic analyses using RAPD for several species of *Anoplophora* and populations of *A. glabripennis* collected from different geographic locations of China and the US.

2 MATERIALS AND METHODS

2.1 Specimen collection

Specimens of five *Anoplophora* species: *A. glabripennis* (Motschulsky), *A. nobilis* (Ganglbauer), *A. chinensis* (Forster), *A. horsfieldi* (Hope), *A. freyi* (Breuning) and eight *A. glabripennis* geographic populations (six from China, two from the United States) were used in this study (Table 1).

Table 1 Collection data for *Anoplophora* species and *A. glabripennis* populations

Sample code	Species	Status	Collection data	Host plant	Preservation
A	*A. chinensis*	Adult	Fujian, China; ZHU Hong-Bin; 2001	*Platanus* sp.	70% ethanol
B	*A. glabripennis*	Adult	Jingyuan, Gansu, China; GAO Rui-Tong; 2000	*Populus* sp.	Live
C	*A. nobilis*	Adult	Jingyuan, Gansu, China; GAO Rui-Tong; 2000	*Populus* sp.	Live
D	*A. horsfieldi*	Adult	Chongqing, China; JIANG Shu-Nan; 1984	*Ulmus* sp.	Dried
E	*A. freyi*	Adult	Sichuan, China; JIANG Shu-Nan; 1986	*Salix* sp.	Dried
F	*A. glabripennis*	Adult	New York, USA; WANG Bao-De; 2001	*Acer saccharum*	70% ethanol
G	*A. glabripennis*	Adult	Chicago, USA; WANG Bao-De; 2001	*Acer saccharum*	70% ethanol
H	*A. glabripennis*	Adult	Qingdao, Shandong, China; AN Yu-Lin; 1999	*Salix* sp.	Live
I	*A. glabripennis*	Adult	Qinglongxia, Ningxia, China; WANG Yue-Jin; 2000	*Populus* sp.	70% ethanol
J	*A. glabripennis*	Adult	Langfang, Hebei, China; GAO Rui-Tong; 2000	*Populus* sp.	Live
K	*A. glabripennis*	Larva	Xi'an, Shaanxi, China; AN Yu-Lin; 1999	*Populus* sp.	−20℃ freeze
L	*A. glabripennis*	Adult	Bameng, Nei Mongol, China; YAN Ao-Jing; 2000	*Populus* sp.	Dried
M	*A. nobilis*	Adult	Qinglongxia, Ningxia, China; WANG Yue-Jin	*Populus* sp.	70% ethanol
N	*Tetropium* sp.	Larva	China	*Pinus* sp.	Dried

2.2 DNA extraction

Either muscle tissues of adult prothorax or integument of individual larvae were taken and washed with 0.9 % NaCl. The materials were then put in a glass tube, and ground with 1.0 mL SDS buffer (20 mmol/L Tris-HCl pH 7.4; 20 mmol/L EDTA; 0.5% SDS). The ground materials were placed into a 2 mL Eppendoff tube along with 30 μL of 20 mg/mL proteinase-K and were incubated at 55℃ over night to digest the materials. The solution was then centrifuged at 12 000 r/min for 5 min. The supernatant liquid was vortexed with equal volume of phenol and chloroform: isoamyl alcohol and centrifuged at 12 000 r/min for 10 min. The upper phase was taken and placed in a new tube. This step was repeated once. Samples were extracted with 1 mL chloroform: isoamyl followed by centrifugation at 12 000 r/min for 10 min. The supernatant, along with 2 volume of ethanol and 0.1 volume 3 mol/L NaAc (pH 5.2), was then vortexed, and precipitated for DNA at −20℃ for at least two hours, followed by centrifugation at 12 000 r/min for 10 min. The precipitate was washed with 300 μL 70% ethanol. This was followed by centrifugation at 12 000 r/min for 10 min. The ethanol was discarded, and the tube with materials in it was dried at room temperature or dried in hot-air oven for 5 min, re-dissolved in 50 μL 1 × TE, and stored in a refrigerator at −20℃. Each DNA sample (density: 10 − 20 ng/μL) was diluted to the density equal to ddH_2O, and 2 L were taken for PCR reactions.

2.3 Primer selection

We selected 51 random primers (20 OPH, 20 OPL, 11 OPQ kits) produced by Operon Technologies Inc. (Alameda, CA, USA). In primer group Ⅰ, 26 of the 40 primers (Table 2) produced polymorphic bands in *Anoplophora* species and *A. glabripennis*

populations. In group Ⅱ, 19 of the 31 primers (Table 3) that we used produced polymorphic bands in phylogeny analyses of *A. glabripennis* population analyses.

Table 2 Group Ⅰ polymorphic primers used in phylogeny analyses of *Anoplophora* species and *A. glabripennis* populations

Primer	Sequence	Total bands	Polymorphic bands	Percent of polymorphic bands (%)
H-02	5′-TCGGACGTGA-3′	56	43	76.8
H-03	5′-AGACGTCCAC-3′	46	46	100
H-05	5′-AGTCGTCCCC-3′	57	57	100
H-07	5′-CTGCATCGTG-3′	52	52	100
H-08	5′-GAAACACCCC-3′	49	35	71.4
H-09	5′-TGTAGCTGGG-3′	37	37	100
H-11	5′-CTTCCGCAGT-3′	32	32	100
H-12	5′-ACGCGCATGT-3′	64	50	78.1
H-14	5′-ACCAGGTTGG-3′	64	64	100
H-15	5′-ACCAGGTTGG-3′	34	20	58.8
L-06	5′-GAGGGAAGAG-3′	55	43	78.1
L-11	5′-ACGATGAGCC-3′	43	29	67.4
L-13	5′-ACCGCCTGCT-3′	55	41	74.5
L-14	5′-GTGACAGGCT-3′	43	29	67.4
L-15	5′-AAGAGAGGGG-3′	46	46	100
L-17	5′-AGCCTGAGCC-3′	36	23	63.9
Q-01	5′-GGGACGATGG-3′	42	14	33.3
Q-02	5′-TCTGTCGGTC-3′	36	36	100
Q-03	5′-GGTCACCTCA-3′	47	47	100
Q-05	5′-CCGCGTCTTG-3′	78	64	82.1
Q-06	5′-GAGCGCCTTG-3′	74	46	62.1
Q-07	5′-CCCCGATGGT-3′	34	34	100
Q-08	5′-CTCCAGCGGA-3′	62	34	54.8
Q-09	5′-GGCTAACCGA-3′	36	36	100
Q-10	5′-TGTGCCCGAA-3	28	14	50
Q-11	5′-TCTCCGCAAC-3′	83	69	82.5

2.4 RAPD reaction and electrophoresis

RAPD reactions were carried out with a MJ Research 100 PCR Instrument (MJ Research, Inc., Waltham, MA, USA) based on the method developed by Williams *et al*. (1990), and Welsh and McClelland (1990). Amplifications were carried out in a total volume of 25 μL: 2.5 μL of 10 × buffer, 3 μL of 25 mmol/L $MgCl_2$, 2.0 μL of 2.5 mmol/L dNTP, 1.0 μL of primer, 1.0 μL of DNA and 15.5 μL of ddH_2O. The reaction profile was 94℃ for 3 min, followed by the addition of 0.3 μL (contain 1.5 U) tag polymerase buffer, then 94℃ for 3 min, then 45 circles (of 94℃ for 30 sec, 36℃ for 30 sec, 72℃ for 90 sec), and 75℃ for 10 min. The product was stored at 4℃. Aliquots (10 μL) of the amplification products were then electrophoresed at 70 V 100 mA for 40 min through a 1.3 percent Seakem LE agarose gel (100 mL) in 1 × TBE (pH 8.0) buffer containing 5 μL 10 mg/mL ethidium bromide. The PCR products were visualized and photographed over a 312 nm UV light (Kodak EDAS 290, Eastman Kodak Co., New Haven, CT, USA).

Table 3 Group Ⅱ polymorphic primers used in phylogeny analyses of *Anoplophora glabripennis* populations

Primer	Sequence	Total bands	Polymorphic bands	Percent of polymorphic bands (%)
H-07	5′-CTGCATCGTG-3	80	72	90
L-06	5′-GAGGGAAGAG-3′	88	88	100
L-07	5′-AGGCGGGAAC-3′	56	56	100
L-10	5′-TCGGACGTGA-3′	72	72	100
L-11	5′-ACGATGAGCC-3′	56	56	100
L-13	5′-ACCGCCTGCT-3	120	120	100
L-14	5′-GTGACAGGCT-3	88	88	100
L-15	5′-AAGAGAGGGG-3′	72	64	88.9
L-17	5′-AGCCTGAGCC-3	72	72	100
Q-01	5′-GGGACGATGG-3′	72	56	77.8
Q-02	5′-TCTGTCGGTC-3′	80	80	100
Q-03	5′-GGTCACCTCA-3′	96	96	100
Q-05	5′-CCGCGTCTTG-3′	128	128	100
Q-06	5′-GAGCGCCTTG-3′	120	112	93.3
Q-07	5′-CCCCGATGGT-3′	96	96	100
Q-08	5′-CTCCAGCGGA-3′	88	64	72.7
Q-09	5′-GGCTAACCGA-3′	88	80	90.9
Q-10	5′-TGTGCCCGAA-3′	48	40	83.3
Q-11	5′TCTCCGCAAC-3′	96	96	100

2.5 RAPD-PCR data analysis

All bands that were consistently amplified by individual random primers were scored for presence or absence in the species and populations. TFPGA, computer software for analyzing population genetics developed by Mark Miller of Utah State University, was used to produce cladograms and calculate genetic distances.

3 RESULTS

Polymorphic bands (Figs. 1 and 2) of the RAPD products of the 26 selected primers in group Ⅰ (Table 2) were used in analyzing the phylogeny of ALB populations and its related species. However, RAPD products of *Tetropium* sp. did not yield obvious polymorphic bands and therefore, were not analyzed together with the rest of specimens. Based on the TFPGA-generated RAPD cladogram from primer group Ⅰ (Fig. 3), eight *A. glabripennis* geographical populations and two *A. nobilis* populations were grouped in one phylogenic cluster that separated from the other three *Anoplophora* species (*A. chinensis*, *A. horsfieldi*, and *A. freyi*). Six geographical populations

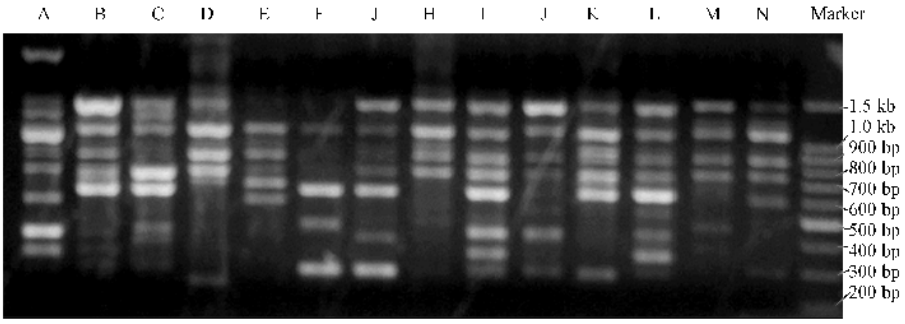

Fig. 1 Photograph of primer OPQ-5 RAPD amplification products showing the genetic variations of *Anoplophora* species and *A. glabripennis* populations

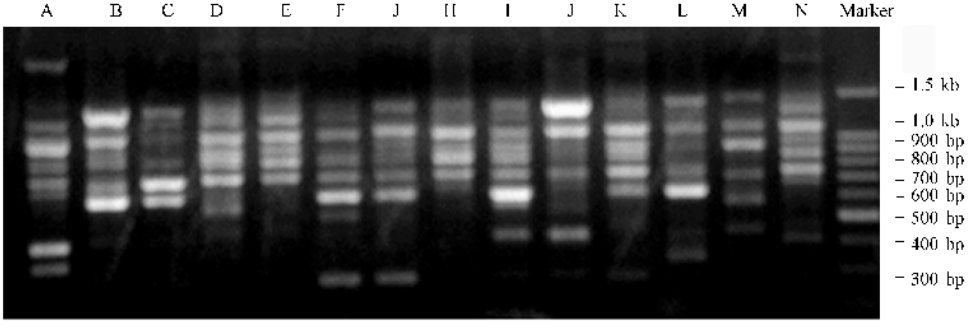

Fig. 2 Photograph of primer OPQ-6 RAPD amplification products showing the genetic variations of *Anoplophora* species and *A. glabripennis* populations

Table 4 Pairwise genetic distance between *Anoplophora* species and *A. glabripennis* populations

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	0												
B	0.6214	0											
C	0.6061	0.1324	0										
D	0.4520	0.5053	0.4651	0									
E	0.4391	0.4917	0.4263	0.1514	0								
F	0.7353	0.3650	0.3531	0.5473	0.5331	0							
G	0.5617	0.3072	0.3185	0.4783	0.4651	0.2525	0						
H	0.4917	0.3650	0.3531	0.2314	0.2632	0.3769	0.3650	0					
I	0.5331	0.3531	0.3650	0.4520	0.4917	0.3891	0.2849	0.2314	0				
J	0.4651	0.3185	0.3299	0.4138	0.4263	0.3531	0.3650	0.3072	0.2525	0			
K	0.5473	0.2960	0.2849	0.2960	0.3769	0.3531	0.3891	0.2211	0.2960	0.2632	0		
L	0.5617	0.2849	0.3185	0.5053	0.5473	0.3650	0.3299	0.3185	0.2007	0.2525	0.2525	0	
M	0.6687	0.3414	0.2849	0.3650	0.3531	0.4263	0.3650	0.1807	0.2960	0.3531	0.2419	0.2960	0

of *A. glabripennis* in China were placed in the same clade, which divided into two branches. The first included specimens of *A. glabripennis* from provinces of Shandong, Ningxia, Shaanxi, Hebei, and Nei Mongol Autonomous Region, as well as *A. nobilis* from Ningxia Hui Autonomous Region. The other branch consisted of *A. glabripennis* and *A. nobilis* from Gansu Province. The two populations of *A. glabripennis* in New York and Chicago grouped in an independent clade with 0.2525 genetic distances (Table 4) between them.

Polymorphic bands (Figs. 4 and 5) of the RAPD products of the 19 selected primers in the group Ⅱ (Table 3) were used to analyze the phylogeny of *A.*

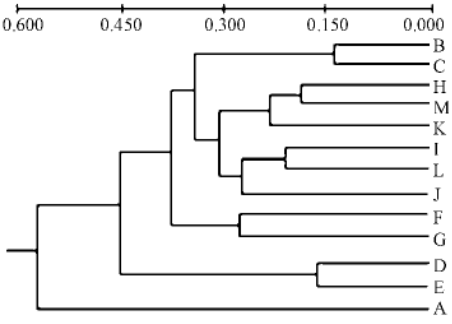

Fig. 3 RAPD cladogram using primer group Ⅰ to show the relationship between *Anoplophora* species and *A. glabripennis* populations generated by UPGMA

glabripennis populations. Based on the RAPD cladogram of primer group Ⅱ (Fig. 6), six geographical populations of *A. glabripennis* in China were grouped in a clade that divided into two branches. Specimens from Ningxia, Hebei, Shandong and Gansu were placed in one branch, while specimens from Nei Mongol, and Shaanxi were placed in the other. *A. glabripennis* populations from New York and Chicago were again in an independent clade with 0.3509 genetic distances between them. This value is less than the genetic distances among *A. glabripennis* populations from the US and China, which ranged from 0.4578 to 0.5960 (Table 5).

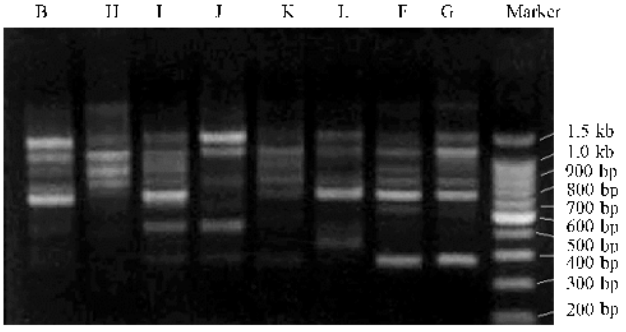

Fig. 4 Photograph of primer Q-6 amplification products showing the genetic variations of *Anoplophora glabripennis* populations

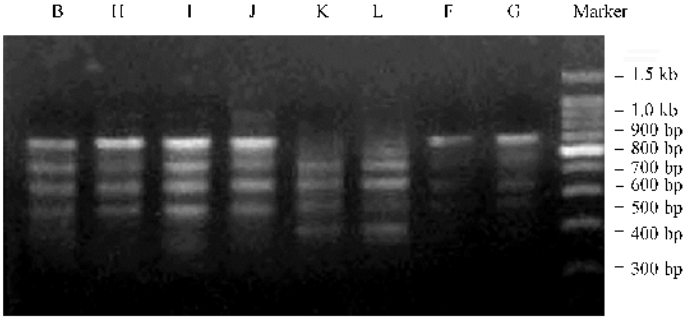

Fig. 5 Photograph of primer L-17 amplification products showing the genetic variations of *Anoplophora glabripennis* populations

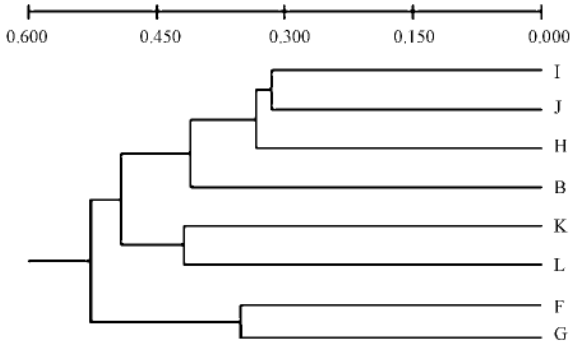

Fig. 6 RAPD cladogram using primer group Ⅱ to show the relationship between *Anoplophora glabripennis* populations generated by UPGMA

Table 5 Pairwise genetic distance between *Anoplophora glabripennis* populations

	B	H	I	J	K	L	F	G
B	0							
H	0.3728	0						
I	0.4183	0.3223	0					
J	0.4418	0.3436	0.3153	0				
K	0.5686	0.4106	0.4578	0.4659	0			
L	0.5419	0.5159	0.4990	0.4741	0.4183	0		
F	0.4990	0.4741	0.5960	0.5686	0.4906	0.5868	0	
G	0.4823	0.4578	0.4906	0.5159	0.5776	0.5868	0.3509	0

4 DISCUSSION

The genetic distances among the five selected species of *Anoplophora* (including the so-called *A. nobilis*) were not consistent with the pre-2001 morphological classification. Specifically, our results indicated that distances among some populations of *A. glabripennis* are greater than the distances that separate a number of paired populations of *A. nobilis* and *A. glabripennis*. This can be seen in the cladogram (Fig. 3), where populations of *A. glabripennis* and *A. nobilis* are intermingled, suggesting that the two are likely a single species. *A. glabripennis* and *A. nobilis*

were previously considered to be separate species based on its morphological characters, host species and geographical distribution (Jiang, 1980; Chen, 1989). Recent studies, however, demonstrated that there is no reproductive separation or isolation between the two, which co-exist in some regions of north and northwest China and have been observed mating freely in field and produced viable offspring (Gao *et al*., 2000). Results of detailed morphological studies in recent years also indicate that *A. nobilis* and *A. glabripennis* are most likely to be the same species (Luo *et al*., 2000; Lingafelter and Hoebeke, 2002).

The genetic distance (0.1514) among specimens from New York City and Chicago is less than that of the specimens of *A. glabripennis* and *A. nobilis* collected from various geographical locations of China. Therefore, New York and Chicago populations are grouped in a cluster that separates them from other *A. glabripennis* population. One reason for this may be that populations of *A. glabripennis* in USA may not be from regions of China where specimens of *A. glabripennis* or *A. nobilis* were collected for this study. *A. glabripennis* (including *A. nobilis*) reportedly also occur in Anhui, Henan, Shanxi and other provinces of China (Wu and Chen, 2003), and in countries such as South Korea and Japan (Gressitt, 1951). There may be other reasons, such as the limitations of RAPD method, which are dominant markers and therefore cannot be used to identify heterozygotes. Further studies are currently underway to include more specimens from all provinces and regions of China and from other parts of Asia where *A. glabripennis* is known to occur. RAPD will be used together with gene sequencing for the genetic analysis of *Anoplophora* species and populations.

Our results indicate that *A. freyi* from Chongqing, China should be grouped together with *A. horsfieldi* collected from Sichuan Province, China in a cluster that separates them from *A. glabripennis* and *A. nobilis* (all locations). This suggests that *A. freyi* indeed exists as a separate species in this part of China (Wu and Jiang, 1998). The genetic distance between *A. chinensis* from Fujian, China and all others is between 0.5 and 0.6 (Fig. 3), indicating a great difference between them.

Acknowledgments This study is a part of a collaborative research project between AQSIQ, CHINA and USDA APHIS, USA. We thank Drs. Larry Hanks and Ken Paige of University of Illinois for their technical help. We are grateful to Mr. ZHU Hong-Bin, Ms. WU Cui-Ping and Mr. SU Han of Jiangsu CIQ for their help. Thanks also to Drs. David Lance and Sue McCombs of USDA Animal and Plant Health Inspection Service for their reviewing of the manuscript.

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光肩星天牛种群间及其近缘种遗传关系的 RAPD 研究

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摘要: 利用 RAPD 技术对采自中国和美国的星天牛属 *Anoplophora* 5 个种及 8 个光肩星天牛 *Anoplophora glabripennis* (Motschulsky)地理种群共 13 个样品进行了遗传相似性分析。选用了 Operon 公司生产的引物 H 系列 20 个, L 系列 20 个, Q 系列 11 个共 51 个引物, 最后从 40 个引物中筛选出 26 个具有多态性的引物作为第一组用于星天牛属种间和光肩星天牛种群间分析, 从 31 个引物中筛选出 19 个具有多态性的引物作为第二组单独用于光肩星天牛种群分析。根据第一组引物实验获得的 RAPD 聚类图及遗传距离表明, 各个地理种群的光肩星天牛和黄斑星天牛 *A. nobilis* 都聚在一起, 形成一个大的分枝, 而四川星天牛 *A. freyi*、棟星天牛 *A. horsfieldi* 和星天牛 *A. chinensis* 均在此分枝之外。来自美国纽约和芝加哥的光肩星天牛种群聚于中国光肩星天牛种群之外的另一个独立的分枝上。分布在我国宁夏、内蒙古和河北的光肩星天牛以及宁夏黄斑星天牛和山东、陕西的光肩星天牛分别聚在一起, 而甘肃的光肩星天牛与甘肃的黄斑星天牛则聚于另一枝, 且它们之间的遗传距离很近, 仅为 0.1324, 说明这两者之间有着极其相近的亲缘关系, 由此推断光肩星天牛和黄斑星天牛的差异很小, 遗传关系难以区分, 进一步证实了它们很可能是同一个种下的两个不同的型。第二组引物实验得到了相似的结果, 来自中国的 6 个光肩星天牛种群全部聚于同一枝中并分成两小枝: 分布于我国宁夏、河北、山东、甘肃的光肩星天牛聚在一起, 内蒙古和陕西的光肩星天牛则成另一枝, 而分布于美国纽约和芝加哥的光肩星天牛仍聚于中国光肩星天牛种群之外的一个单独的分枝上。但是美国光肩星天牛与中国光肩星天牛之间的遗传距离最近的为 0.4578, 最远的为 0.5960。由此认为, 本研究中采自美国的两个光肩星天牛种群的样本和采自中国的光肩星天牛种群的样本之间存在显著差异, 遗传关系较远。有必要从中国和世界其他天牛分布地采集更多样本做进一步 DNA 分析。

关键词: 星天牛属; 光肩星天牛; 黄斑星天牛; 种群; RAPD; 遗传距离
中图分类号: Q969　　**文献标识码**: A　　**文章编号**: 0454-6296(2004)02-0229-07

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